Journal of Medical Virology 59:313–317 (1999)

High Prevalence of TT Virus (TTV) Infection in Patients on Maintenance Hemodialysis: Frequent Mixed Infections With Different Genotypes and Lack of Evidence of Associated Liver Disease

Xavier Forns,[1] Patricia Hegerich,[1] Alejandro Darnell,[2] Suzanne U. Emerson,[1] Robert H. Purcell,[1] and Jens Bukh[1]*

[1]Hepatitis Viruses and Molecular Hepatitis Sections, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland
[2]Nephrology Department, Hospital Clinic i Provincial, University of Barcelona, Barcelona, Spain

Recently, a novel DNA virus, TT virus (TTV), was identified in patients with post-transfusion non-A-G hepatitis. We analyzed the prevalence and clinical implications of TTV infection in a cohort of 96 Spanish patients on long-term hemodialysis. TTV DNA was detected by nested PCR in 51 (53%) of 96 patients, a prevalence significantly higher than that found in healthy blood donors. Persistent liver test abnormalities were found in only 2 (7.7%) of 26 patients infected with TTV alone, compared with 12 (75%) of 16 patients infected with hepatitis C or hepatitis B virus, or both ($P < 0.01$). Mixed infections with multiple strains of TTV, including different major genotypes, were common in patients on hemodialysis. These patients had received a significantly greater number of blood units (22.7 ± 20) compared with patients apparently infected with a single strain of TTV (8.9 ± 11) ($P = 0.01$). Phylogenetic analyses of TTV from infected patients identified strains of genotypes 1, 2, 3, and 4. In summary, TTV infection was common in patients on hemodialysis but was not associated with liver disease ***J. Med. Virol. 59:313–317, 1999.***

KEY WORDS: nosocomial transmission; blood transfusion; hepatitis

INTRODUCTION

Each time a blood-borne virus is discovered, concerns are raised about its possible pathogenicity. Patients on long-term hemodialysis are especially susceptible to parenterally transmitted agents and therefore represent an important population for analysis of the clinical and epidemiological implications of newly identified agents. Recently, a novel DNA virus, TT virus (TTV), was identified in patients with post-transfusion non-A-G hepatitis [Nishizawa et al., 1997]. Transient aminotransferase elevation also occurred in several patients at 6–9 weeks after transfusion of TTV-contaminated blood products [Simmonds et al., 1998]. Similarly, the prevalence of TTV in patients with cryptogenic chronic liver disease and fulminant hepatic failure was significantly higher than in blood donors [Charlton et al., 1998; Naoumov et al., 1998; Simmonds et al., 1998]. Finally, TTV-DNA titers were found to be 10- to 100-fold higher in liver tissue than in serum, suggesting that TTV is hepatotropic [Okamoto et al., 1998a]. However, a clear association between TTV infection and liver disease has not been demonstrated. Thus, Naoumov et al. [1998] found that most TTV-infected patients lacked biochemical or histological evidence of liver disease. Clearly, additional studies are needed to elucidate the natural history of TTV.

TTV is a nonenveloped single-stranded DNA virus (genome length of ≈3.7 kb) that initially was thought to be a distant relative of parvoviruses [Okamoto et al., 1998a] or circoviruses [Takahashi et al., 1998a]. However, recent molecular and biophysical characterization of TTV indicates that this virus has a circular negative-strand DNA genome and could therefore constitute a new virus family [Mushahwar et al., 1999]. The TTV genome exhibits a high degree of genetic heterogeneity; phylogenetic analyses of TTV sequences from around the world indicate that this virus can be

Grant sponsor: National Institutes of Health; Grant number: CO-56000.

The nucleotide sequence data reported herein have been assigned GenBank accession numbers AF 152420–152461.

*Correspondence to: Jens Bukh, Hepatitis Viruses Section, Building 7, Room 201, NIAID, LID, National Institutes of Health, 7 Center Dr MSC 0740, Bethesda, MD 20892-0740. E-mail: jbukh@niaid.nih.gov

Accepted 6 April 1999

classified into different genotypes and probably into numerous subtypes [Höhne et al., 1998; Mushahwar et al., 1999; Okamoto et al., 1998a,b; Simmonds et al., 1998; Tanaka et al., 1998; Viazov et al., 1998]. The prevalence of TTV in the general population is high, ranging from 1.9% in Scottish blood donors to as high as 70% in rural areas of Papua, New Guinea [Okamoto et al., 1998a; Prescott and Simmonds, 1998; Simmonds et al., 1998]. TTV is transmitted by the parenteral route [Okamoto et al., 1998a; Simmonds et al., 1998]; recent data suggest that TTV can also be transmitted by the fecal-oral route [Okamoto et al., 1998b].

In the present study, we analyzed the prevalence of TTV in Spanish patients on long-term hemodialysis, the potential risk factors associated with TTV transmission and the relevance of the virus as a causative agent of liver disease in this cohort. We also analyzed the genetic diversity of TTV in this group of patients.

PATIENTS AND METHODS

Patients

Ninety-six patients on maintenance hemodialysis for end-stage renal disease were included in the study [Forns et al., 1997a]. All patients were treated at the same hospital in Barcelona, Spain. Patients are treated in three different units depending on various criteria [Forns et al., 1997a]. Dialyzers are not reused, and the dialysate circuit is disinfected with a solution of sodium hypochlorite after each individual session of hemodialysis.

Routine liver tests [aminotransferases (ALT, AST), bilirubin, γ-glutamyl transferase (GGT) and alkaline phosphatase], as well as tests for antibody to hepatitis C virus (HCV, anti-HCV) and for hepatitis B virus (HBV) surface antigen (HBsAg) were performed every 6 months. An abdominal ultrasonographic examination was performed every year. The diagnosis of liver cirrhosis was defined by clinical, biochemical, and ultrasonographic criteria or by histologic criteria when a liver biopsy was available [Forns et al., 1997a]. Time on hemodialysis and the history of blood transfusion and surgical procedures were recorded to evaluate their role as risk factors for transmission of TTV.

DNA Extraction and PCR

A total of 100 μl of serum was incubated with 400 μl of a solution containing 50 mM Tris-HCl, 1 mM EDTA, 10 mM NaCl, 0.5% sodium dodecyl sulfate (SDS) and 1 mg/ml of proteinase K (Gibco-BRL, Gaithersburg, MD) for 1 hour at 65°C. Thereafter, 500 μl of phenol:chloroform/isoamyl alcohol (25:24:1) (Gibco-BRL) was added, and the mixture was centrifuged at 14,000 rpm for 15 min at 4°C. After recovery of the aqueous phase, 1/50 vol of 3 M sodium acetate, 2 vol of ice-cold ethanol, and 1 μl of glycogen (Boehringer-Mannheim, Indianapolis, IN) were added. The mixture was incubated overnight at –20°C and centrifuged at 14,000 rpm for 20 min at 4°C. The pellet was washed with 1 ml of 70% ethanol. For DNA amplification by polymerase chain reaction (PCR), the pellet was resuspended in 100 μl of master mix: 10 μl of 10× PCR buffer (Perkin Elmer, Foster City, CA), 10 μl of 25 mM $MgCl_2$ (Perkin Elmer), 5 μl of 10 μM external sense primer (CAT TCA CAG ACA GAG GAG AAG G), 5 μl of 10 μM external antisense primer (GTT GGA TAC CAT TTA GCT CTC ATT), 2 μl of 10 mM dNTP (Boehringer-Mannheim), 0.5 μl of AmpliTaq Gold DNA polymerase (Perkin Elmer), and 67.5 μl of water.

The first round of PCR amplification was performed for 35 cycles with denaturation at 94°C for 1 min (initial denaturation step of 12 min), annealing at 45°C for 2 min, and amplification at 72°C for 3 min. For the second round of PCR amplification, a 10-μl aliquot of the first PCR reaction was added to a master mix consisting of 9 μl of 10× PCR buffer, 9 μl of 25 mM $MgCl_2$, 5 μl of 10 μM internal sense primer [NG061(Okamoto et al., 1998a), GGC AAC ATG TTA TGG ATA GAC TGG], 5 μl of 10 μM internal antisense primer (CCT GGC ATT TTA CCA TTT CCA AA), 2 μl of 10 mM dNTP, 0.5 μl of AmpliTaq Gold DNA polymerase, and 59.5 μl of water. The same cycling conditions were used for the second round of PCR. As a positive control, we used 100 μl of a 10^{-2} dilution of a serum sample from a TTV-positive patient with a titer of 10^3 genome equivalents/ml (genotype 1a), as determined by hemi-nested PCR [Okamoto et al., 1998a]. In the modified nested-PCR assay, we found this sample to have a titer of 10^3–10^4 genome equivalents/ml. One negative control was introduced for every four samples tested. PCR products were analyzed in agarose gels stained with ethidium bromide; specificity of DNA bands of the expected size was confirmed by sequence analysis (see below). Sequence analysis demonstrated that nonspecific DNA amplification occurred in five patients in whom a weak DNA band of the expected size appeared after nested PCR.

Sequence Analyses

Both strands of DNA from nested PCR products [225 nts, excluding the primers, position 1939–2163 from TA278 (GenBank accession #AB008394)] [Okamoto et al., 1998a] were purified by the Wizard PCR Preps DNA purification kit (Promega, Madison, WI) and sequenced with the PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) by using Taq DNA polymerase (Perkin Elmer). Sequences were analyzed with CLUSTAL W (version 1.6) and PAUP (version 4.0, created by D.L. Swafford, Laboratory of Molecular Systematics, Smithsonian Institution, Washington, DC), both available through the GCG Sequence Analysis Package (version 9.1; Genetics Computer Group, Madison, WI).

Cloning

PCR products were cloned into the pCR2.1 vector by using the original TA cloning kit (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Both DNA strands of clones containing the insert of the expected size were sequenced.

TABLE I. Risk Factors for Acquisition of Hepatitis Viruses and Presence of Persistent Liver Test Abnormalities According to Infection Status*

	Age (yr)	Time on HD[b] (yr)	Transfused (%)	Blood units	Surgery (%)	Persistent liver test abnormalities[c]
A. No HCV/HBV/TTV (n = 29)	60 ± 15	3.7 ± 3.2	19 (66%)	11 ± 19	21 (72%)	2 (6.9%)
B. TTV (n = 26)	59 ± 17	4.2 ± 3.2	22 (85%)	11 ± 13	16 (62%)	2 (7.7%)
C. HBV and/or HCV (n = 16)	56 ± 16	6 ± 3.7	14 (87%)	30 ± 58[a]	12 (81%)	12 (75%)
D. TTV + HBV and/or HCV (n = 25)	57 ± 18	9 ± 6	17 (68%)	14 ± 17	19 (76%)	16 (64%)

*HGV infection status was not considered in the analysis. Quantitative variables are expressed as mean ± SD.
[a]One patient received more than 200 blood units.
[b]Time on hemodialysis was significantly shorter in patients from groups A and B compared with patients from groups C and D ($P < 0.01$).
[c]Abnormal liver tests lasting more than 1 year were significantly more frequent in patients from groups C and D compared with patients from groups A and B ($P < 0.01$).

Statistical Analysis

Quantitative variables are expressed as mean ±SD. For categorical variables, comparisons between groups were made by the Fisher's exact test or by the chi-square test. For quantitative variables, comparisons between groups were made by Student's *t*-test and by one-way analysis of variance (ANOVA), or by a non-parametric test, when necessary.

RESULTS

Serum TTV DNA was detected by nested PCR in 51 (53.1%) of 96 patients on hemodialysis. This prevalence is significantly higher than in blood donors from the same geographical area, where TTV was detected in 23 (13.7%) of 168 individuals ($P < 0.001$) [Giménez-Barcons et al., 1999].

To analyze the potential risk factors for TTV acquisition, as well as the impact of TTV infection on liver disease, we divided the patients into four groups: (A) patients not infected with HBV, HCV, or TTV; (B) patients infected only with TTV; (C) patients infected with HBV or HCV, or both, but not with TTV; and (D) patients infected with TTV and with HBV or HCV, or both. Hepatitis G virus (HGV) infection status was not considered in the analysis because we previously showed that this agent is not a cause of liver disease in this cohort of patients [Forns et al., 1997a]. The four groups were compared in terms of age, time on hemodialysis, transfusion of blood products, and history of major surgery (Table I). No significant differences were observed among these groups regarding age, blood transfusion, and surgery. However, patients not infected with HBV or HCV, or both (groups A and B) had been on hemodialysis for a significantly shorter period of time than patients infected with either or both of these viruses (groups C and D) ($P < 0.01$).

Abnormal liver tests lasting more than 1 year were recorded in 32 patients (Table I). Patients infected with HBV or HCV, or both (groups C and D) had a significantly higher prevalence of persistent liver test abnormalities [28 (68%) of 41 patients] compared with patients infected with TTV only or with none of these viruses (groups A and B) [4 (7%) of 55 patients] ($P < 0.001$). Remarkably, only 2 (7.7%) of 26 patients infected solely with TTV (group B) had repeatedly abnormal liver tests; two additional patients presented a mild elevation of liver enzymes (ALT in one case and GGT in the other) in one of their biannual biochemical evaluations. These data did not differ from those of patients not infected with HBV, HCV, or TTV (group A): 2 (6.9%) of 29 patients had repeatedly abnormal liver tests. None of the four cases of liver cirrhosis diagnosed among the entire cohort of patients belonged to groups A or B.

Sequence data were obtained from all 51 TTV-positive patients. The TTV sequences from all isolates were 225 nts in length, without insertions or deletions, and encoded 75 amino acids. Multiple double peaks in the sequence chromatograms strongly suggested the coexistence of more than one TTV strain in 14 (27%) of the 51 patients analyzed. In these cases, it was not possible to determine a consensus sequence at several nucleotide positions. In order to confirm the existence of mixed infections, the PCR bands from two representative patients (HD1 and HD20) were cloned; 10 and 9 clones, respectively, from the patients were sequenced. Patients HD1 and HD20 were co-infected with two and three different TTV strains, respectively. Interestingly, two major genotypes coexisted in each patient (Fig. 1). We observed that the 14 patients infected with more than one TTV strain had received a significantly greater number of blood units (22.7 ± 20) than patients apparently infected with a single TTV strain (8.9 ± 11) ($P = 0.01$).

The identity at the nucleotide level among the analyzed TTV isolates ranged from 63% to as high as 99%. Review of the medical records of patients with the highest degree of homology showed that they were dialyzed in the same unit of the hospital, suggesting nosocomial transmission.

A phylogenetic analysis of the TTV genomic sequences from 15 published TTV reference strains and TTV sequences from our patients showed that the sequences segregated into at least six major groups (Fig. 1). Phylogenetic analysis of the deduced amino acid sequences supported the division of TTV into multiple genotypes (data not shown). Among the 37 TTV-infected patients with a clear consensus PCR sequence, the genotype distribution was as follows: genotype 1, 8 (21.7%) patients; genotype 2, 20 (54%) patients; genotype 3, 3 (8.1%) patients; and genotype 4, 6 (16.2%) patients.

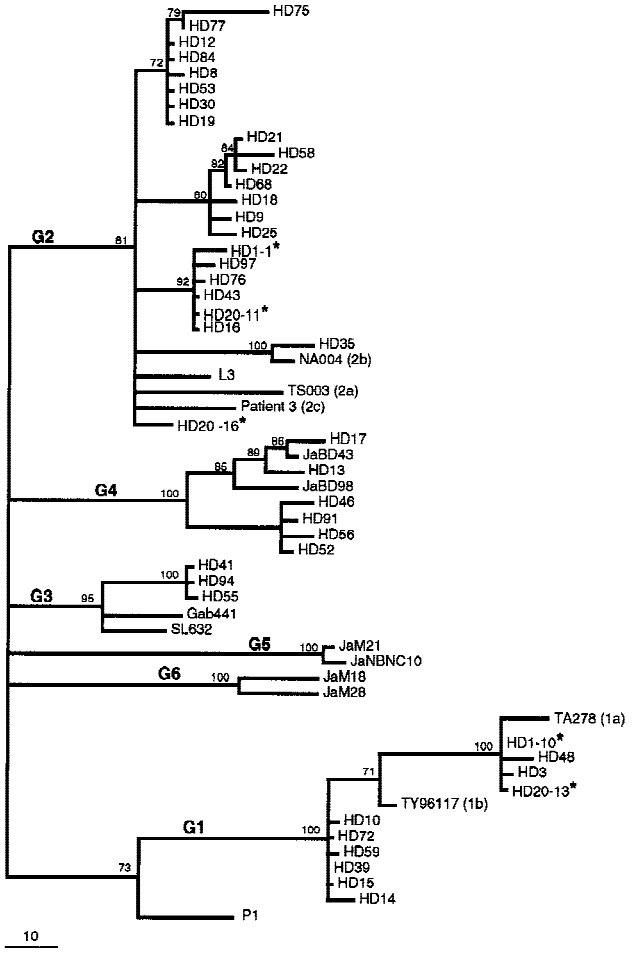

Fig. 1. Genetic analysis of TT virus (TTV) among Spanish hemodialysis patients. Analysis was based on a 222-nucleotide fragment, spanning nts 1939–2160 of the TTV genome [strain TA278, Okamoto et al., 1998a]. The analysis includes TTV sequences from 15 reference strains [Höhne et al., 1998; Okamoto et al., 1998a; Takahashi et al., 1998a; Tanaka et al., 1998; Viazov et al., 1998] and from 37 of 51 TTV-positive hemodialysis patients (HD). Two and three clones from patients HD1 and HD20, respectively, among 14 patients with mixed TTV infection, are also included in the analysis and are depicted with an asterisk. Genotype designations assigned previously [Höhne et al., 1998; Naumov et al., 1998; Okamoto et al., 1998a; Tanaka et al., 1998] are indicated. Phylogenetic trees were constructed by aid of PAUPSEARCH and PAUPDISPLAY from the PAUP computer software package (version 4.0). Bootstrap 70% majority-rule consensus trees (midpoint rooting) were obtained by performing heuristic search (optimality criterion, maximum parsimony; all characters equal weight; 1,000 replicates) and displayed by aid of TREEVIEW (version 1.5) [Page, 1996]. Bootstrap values of >70% are indicated.

DISCUSSION

In this study, we have shown that TTV infection is highly prevalent in patients on maintenance hemodialysis. The prevalence might have been underestimated because TTV has a high degree of genetic heterogeneity that could confound PCR amplification [Okamoto et al., 1998a; Simmonds et al., 1998; Takahashi et al., 1998a,b]. In the present study, we used conserved sets of nested primers in the PCR assay, based on previously published sequence data [Okamoto et al., 1998a]. This assay detected TTV DNA in patients infected with four major TTV genotypes.

Regarding the analysis of epidemiological variables, time on hemodialysis was considerably shorter for patients infected only with TTV compared with patients infected with HBV or HCV, or both. This finding suggests that once patients enter a hemodialysis program, contact with TTV occurs rapidly. As supported by the results of this study, transfusion of blood products is likely to be a relevant mechanism for the transmission of multiple TTV strains in this group of patients. Not all TTV-infected patients had a history of blood transfusion or major surgery, however. Since TTV exhibits extensive genetic heterogeneity, the finding of closely related TTV sequences in patients who had been dialyzed in the same unit suggests nosocomial transmission. Nosocomial transmission by the parenteral route happens in patients on hemodialysis but seems to occur only sporadically in our units [Forns et al. 1997b; Forns et al., 1997c]. Nosocomial transmission of TTV by other mechanisms, such as the fecal-oral route [Okamoto et al., 1998b] should be considered in these patients.

Whether TTV causes liver disease remains uncertain [Charlton et al., 1998; Giménez-Barcons et al., 1998; Naoumov et al., 1998; Simmonds et al., 1998a], but it seems unlikely that an agent so prevalent in healthy blood donors would be an important cause of hepatitis. We have shown that abnormal liver tests were uncommon in hemodialysis patients infected with TTV alone, in contrast to patients infected with known hepatotropic viruses such as HBV and HCV. Thus, our results do not support a role for TTV as a causative agent of liver disease in hemodialysis patients. However, our data do not exclude the possibility that mild and transient abnormalities in liver enzymes detected in a few patients were caused by TTV infection.

The fact that hemodialysis patients are polytransfused makes it likely that they are at risk of multiple exposures. Therefore, it was interesting that a significant number of patients were apparently coinfected with different strains of TTV. Sequence analysis of clones from two patients with apparent mixed infections showed that TTV strains belonging to two different major genotypes could coexist in a single patient. Our results suggest that infection with one TTV type does not protect against infection with another TTV type.

Sequence analysis confirmed that the TTV genome contains a high degree of genetic variability. Phylogenetic analysis of TTV DNA sequences show that in Spain, TTV genotypes 1, 2, 3, and 4 are present among TTV-infected patients. Worldwide, at least six major genotypes have been identified to date [Höhne et al., 1998; Naumov et al; 1998; Okamoto et al., 1998a,b; Takahashi et al., 1998a; Tanaka et al., 1998]. Our analysis, as well as previous analyses, also suggest the exsistence of multiple subtypes in genotypes 1, 2 and 4 (Fig. 1); however, longer genomic fragments must be sequenced to confirm these data.

In summary, our results show that TTV infection is very prevalent in patients on long-term hemodialysis.

However, TTV does not appear to be a cause of chronic liver disease in these patients.

ACKNOWLEDGMENTS

Informed consent to conduct this study was obtained from all patients. We thank Dr. Mayumi for kindly providing the positive control sample and staff members at SAIC (Frederick, MD) for assistance in performing sequence reactions. Computer-assisted sequence analyis was through the NIH Helix System, DCRT, NIH.

REFERENCES

Charlton M, Adjei P, Poterucha J, Zein N, Moore B, Therneau T, Krom R, Wiesner R. 1998. TT-virus infection in North American blood donors, patients with fulminant hepatic failure, and cryptogenic cirrhosis. Hepatology 28:839–842.

Forns X, Fernández-Llama P, Costa J, Lopez-Labrador FX, Ampurdanes S, Olmedo E, Saiz JC, Guilera M, Lopez-Pedret J, Sanchez-Tapias JM, Darnell A, Jimenez de Anta MT, Ordinas A, Rodes J. 1997a. Hepatitis G virus infection in a hemodialysis unit: prevalence and clinical implications. Nephrol Dial Transplant 12:956–960.

Forns X, Fernández-Llama P, Pons M, Costa J, Ampurdanes S, Lopez-Labrador FX, Olmedo E, Lopez-Pedret J, Darnell A, Revert Ll, Sanchez-Tapias JM, Rodes J. 1997b. Incidence and risk factors of hepatitis C virus infection in a haemodialysis unit. Nephrol Dial Transplant 12:736–740.

Forns X, Tan D, Alter HJ, Purcell RH, Bukh J. 1997c. Evaluation of commercially available and in-house reverse transcription-PCR for detection of hepatitis G virus or GB virus C. J Clin Microbiol 35:2698–2702.

Giménez-Barcons M, Forns X, Ampurdanès S, Guilera M, Soler M, Soguero C, Sanchez A, Mas A, Bruix J, Sanchez-Tapias JM, Rodes J, Saiz JC. 1999. Infection with a novel human DNA virus (TTV) has no pathogenic significance in patients with liver diseases. J Hepatol 30:1028–1034.

Höhne M, Berg T, Müller AR, Schreier E. 1998. Detection of sequences of TT virus, a novel DNA virus, in German patients. J Gen Virol 79:2761–2764.

Mushahwar IK, Erker JC, Muerhoff AS, Leary TP, Simons JN, Birkenmeyer LG, Chalmers ML, Pilot-Matias TJ, Desai SM. 1999. Molecular and biophysical characterization of TT virus: evidence for a new virus family infecting humans. Proc Natl Acad Sci USA 96:3177–3182.

Naoumov NV, Petrova EP, Thomas MG, Williams R. 1998. Presence of a newly described human DNA virus (TTV) in patients with liver disease. Lancet 352:195–197.

Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M. 1997. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem Biophys Res Commun 241:92–97.

Okamoto H, Nishizawa T, Kato N, Ukita M, Ikeda H, Iizuka H, Miyakawa Y, Mayumi M. 1998a. Molecular cloning and characterization of a novel DNA virus (TTV) associated with posttransfusion hepatitis of unknown etiology. Hepatol Res 10:1–16.

Okamoto H, Akahane Y, Ukita M, Fukuda M, Tsuda F, Miyakawa Y, Mayumi M. 1998b. Fecal excretion of a nonenveloped DNA virus (TTV) associated with posttransfusion non-A-G hepatitis. J Med Virol 56:128–132.

Page RDM. 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358.

Prescott LE, Simmonds P. 1998. Global distribution of transfusion-transmitted virus. N Engl J Med 339:776–777.

Simmonds P, Davidson F, Lycett C, Prescott LE, MacDonald DM, Ellender J, Yap PL, Ludlam CA, Haydon GH, Gillion J, Jarvis LM. 1998. Detection of a novel DNA virus (TTV) in blood donors and blood products. Lancet 352:191–195.

Takahashi K, Ohta Y, Mishiro S. 1998a. Partial ~ 2.4-kb sequences of TT virus (TTV) genome from eight Japanese isolates: diagnostic and phylogenetic implications. Hepatol Res 12:111–120.

Takahashi K, Hoshino H, Ohta Y, Yoshida N, Mishiro S. 1998b. Very high prevalence of TT virus (TTV) infection in general population of Japan revealed by a new set of PCR primers. Hepatol Res 12: 233–239.

Tanaka Y, Mizokami M, Orito E, Ohno T, Nakano T, Kato T, Kato H, Mukaide M, Park Y-M, Kim B-S, Ueda R. 1998. New genotypes of TT virus (TTV) and a genotyping assay based on restriction fragment length polymorphism. FEBS Lett 437:201–206.

Viazov S, Ross RS, Niel C, de Oliveira JM, Varenholz C, Da Villa G, Roggendorf M. 1998. Sequence variability in the putative coding region of TT virus: evidence for two rather than several major types. J Gen Virol 79:3085–3089.